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In Vitro Complement-Binding on Cytoplasmic Structures in Normal Human Skin: I. Immunofluorescence Studies

H. HINTNER, G. STINGL, G. SCHULER, AND K. WOLFF

Department of Dermatology, University of Innsbruck, Innsbruck, Austria (HH,GSch) and Department of Dermatology I, University of Vienna, Vienna, Austria (GSt, KW)

Incubation of cryostat sections of normal human skin with normal human serum (NHS) at 37°C followed by fluorescein isothiocyanate labeled rabbit antihuman C3 (FITC-R/Hu-C3) yields cytoplasmic staining of various cell types including keratinocytes of the upper epidermal layers, melanocytes, fibroblasts, smooth muscle cells, and cells lining vascular structures.

Deposition of C3 on the respective cytoplasmic structures is most likely due to activation of the classical complement (C) cascade on these structures since no fluorescent staining is observed when serum of patients with hereditary C4-deficiency is used instead of NHS or when incubation with NHS is performed in the presence of EDTA or EGTA in concentrations known to inhibit classical C pathway activation. Further evidence suggesting the involvement of the classical C pathway comes from the finding that incubation of cryostat skin sections with NHS followed by FITC labeled rabbit antihuman Clq (FITC-R/Hu-Clq) results in a fluorescent staining pattern remarkably similar to that seen after exposure of cryostat skin sections to NHS and FITC-R/Hu-C3.

Although formal proof is lacking, our investigations strongly indicate that binding to and activation of C components on cytoplasmic structures occur independently of the presence of circulating antibodies. This assumption is based on the finding that in 17 out of 20 NHS we were not able to detect any skin reactive antibodies by indirect immunofluorescence (IF) techniques. More conclusive evidence for a direct, antibody-independent interaction between C components and cytoplasmic structures is provided by the observation that incubation of the substrate with purified Clq followed by FITC-R/Hu-Clq results in cytoplasmic staining of some of the skin cell populations described above.

The phenomenon of C-binding and activation on cytoplasmic structures of normal human skin cells may be a critical event in the initiation of complement mediated pathophysiological reactions of the skin.

Immune complex-triggered activation of the complement cascade leads to the formation of biologically active fragments which have the capacity to bind to cell surface receptor sites specific for a particular fragment (reviewed in reference 1). More recent evidence indicates that the binding of Clq to cells or cell constituents may occur independently of its previous interaction with complexed antibody in that for example unaltered Clq may directly bind to bacterial lipopolysaccharides [2], the envelope of oncorna viruses [3], heart subcellular membranes [4], glutaraldehyde-treated red cells [5], surface antigens of *Trypanosoma brucei* [6] and DNA [7]. Recently, Linder and co-workers have presented evidence which indicates that C-activation can occur on cytoplasmic structures of various cell types including cytoskeletal intermediate-sized filaments of cultured human embryonal fibroblasts and human fetal connective tissue [8], of muscle cells and vascular endothelium [9,10].

In the present study we have obtained evidence with the use of IF techniques that components of the classical C-pathway can be demonstrated in an intracytoplasmic location on cryostat sections of normal human skin previously exposed to fresh NHS. Cell types exhibiting the C-fixing structures include keratinocytes of the upper epidermal layers, melanocytes, fibroblasts, endothelial cells and smooth muscle cells. For at least 2 cell populations, i.e., keratinocytes of the upper epidermal layers and smooth muscle cells, we have suggestive evidence that Clq, after binding to cytoplasmic structures, initiates the activation of the classical C pathway and that this event can occur independently of antibody activity.

MATERIALS

Sources of antisera and reagents: FITC-R/Hu-C3, FITC-labeled rabbit antiserum against human IgA or fibrinogen (all sera F/P ratio 2,3; DAKO antibody titre 100 μg/ml): Dako Immunoglobulins, Copenhagen, Denmark.

FITC-R/Hu-Clq (F/P ratio 1,7; 12 ± 5 mg/ml protein with 10 ± 5% specific antibody content) and antisheep erythrocyte antibodies: Behringwerke AG, Marburg, FRG. FITC-labeled rabbit antiserum against human IgG (F/P ratio 3,5; 1,7 mg antibody/ml) or IgM (F/P ratio 3,4; 1,5 mg antibody/ml): Kent Laboratories Ltd., North Vancouver, BC, Canada.

Chemicals: Tissue-Tek II O.C.T. compound: Lab-Tek Products, Division Miles Laboratories Inc., Naperville, Ill. Ethidium bromide and EGTA: Sigma Chemical Co., St. Louis, Mo. EDTA: Titriplex III, Merck, Darmstadt, FRG. Immunodiffusion plates: Quantiplate, Biotest-Serum-Institute GmbH, Frankfurt, FRG. Functionally purified C-components: Cordis Laboratories, Miami, Fla. Disposable dermatome: Davol Inc., Providence, R.I. Cryo-cut microtome: American Optical Corporation. Fluorescent microscope: Orthoplan Universelles Großfeldmikroskop, Leitz, Wetzlar.

METHODS

Normal human skin was obtained from 10 healthy donors by punch biopsy or with the aid of a disposable dermatome following local

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Reprint requests to: Dr. H. Hintner, Universitätsklinik für Dermatologie und Venerologie, Anichstr. 35, A-6020 Innsbruck, Austria.

Abbreviations:
C: complement
EDTA: ethylenediaminetetraacetic acid
EGTA: ethyleneglycoltetraacetic acid
FITC-R/Hu-C3: fluorescein isothiocyanate labeled rabbit antihuman C3
FITC-R/Hu-Clq: fluorescein isothiocyanate labeled rabbit antihuman Clq
F/P ratio: fluorescein/protein ratio
IF: Immunofluorescence
NHS: normal human serum
PBS: phosphate-buffered saline
U-Cyt antigens: upper cytoplasmic antigens

anesthesia with 2% lidocaine. The skin specimens were embedded in Tissue-Tek II O.C.T. compound, snap frozen in liquid nitrogen and stored at −70°C until sectioning. 4 μm cryostat sections were air-dried for 20 min and then washed in phosphate buffered saline (PBS), pH 7.3, for 10 min. Skin sections were then incubated for 30 min at 37°C with a 1:10 dilution of either freshly drawn human serum or samples which had been stored at −70°C and thawed immediately before use. Sera from 20 normal healthy individuals and from 2 patients with hereditary deficiency of the 4th component of C were used. Evidence for the complete absence of C4 in the respective sera was obtained by single radial immunodiffusion [11] using commercially available immunodiffusion plates and a hemolytic assay as described by Lachman and Hobart [12] using antisheep erythrocyte antibodies and functionally purified complement components.

After washing 3 times in PBS for 10 min, the specimens were overlayered with FITC-R/Hu-C3 or FITC-R/Hu-Clq for 30 min at 37°C. After rinsing the slides with PBS three times for 10 min, they were mounted with glycerine/PBS (4:1) and viewed under a fluorescent microscope equipped for incident illumination. In order to better distinguish the dermal-epidermal junction and thus to determine whether stained cells at the junction were intraepidermal or in the dermis, nuclear counterstaining was performed with 0.001% ethidium bromide. For controls, test sera were substituted by PBS. For the detection of circulating antibodies possibly directed against cytoplasmic antigens in various skin cells, FITC-labeled rabbit-antihuman immunoglobulin or FITC-labeled rabbit antihuman fibrinogen were used following the incubation with 1:5 or 1:10 diluted NHS.

In several experiments purified Clq in a concentration of 100 μg/ml was used in a first incubation period followed by FITC-R/Hu-Clq. The Clq component was isolated from fresh normal human serum by a slight modification [13] of the method described by Volanakis and Stroud [14]. Briefly, the purification procedure consists of 2 successive precipitation steps at low ionic strength in the presence of EDTA in order to dissociate Clr and Cls. 10 ml of 0.1 M EDTA, pH 7.5, were added to 40 ml of fresh human serum. After incubation at 37°C for 10 min the serum was chilled to 0°C and 200 ml of 0.005 M EDTA were added slowly. The mixture was allowed to stand at 0–3°C for 1 hr with occasional stirring. The resulting precipitate was recovered by centrifugation at 12,000 g for 30 min, washed twice with 0.022 M EDTA, pH 7.5 and dissolved in 10 ml 0.75 M NaCl, 0.01 M EDTA, pH 5.0. This solution was stored for 18 hr at 4°C and then centrifuged at 30,000 g for 30 min at 4°C to remove insoluble material. The supernatant was dialyzed for 4 hr against 2 changes of 0.067 M EDTA, pH 5.0. The resulting precipitate was collected by centrifugation at 12,000 g for 30 min, washed twice with 20 ml dialyzing buffer and then dissolved in 3 ml 0.3 M NaCl, 0.01 M EDTA, pH 7.5. After a 2-hr incubation at 4°C, insoluble aggregates were removed by centrifugation at 30,000 g for 30 min at 4°C. After determination of the protein concentration, the presence of detectable amounts of immunoglobulins was excluded by Ouchterlony immunodiffusion techniques [15].

In order to obtain information about a possible activation of the classical complement pathway after binding of Clq, test sera were treated with either EDTA or EGTA in a final concentration of 10 mM. This concentration of EDTA is known to block both the classical and the alternative C-pathway; 10 mM EGTA has been shown to inhibit only the classical C-pathway [16].

RESULTS

Detection of Skin-reactive Circulating Antibodies in Normal Human Sera

When cryostat sections from normal human skin were overlayered with a 1:5 dilution of any of the 20 test sera obtained from healthy individuals and then reacted with FITC-labeled antisera to human IgG, a homogenous cytoplasmic staining of keratinocytes in the upper epidermal layers was seen with 3 of the test sera. This positive reactivity seen in the epidermis contrasted with a complete lack of staining within the dermis. The staining pattern observed was indistinguishable from that described by other authors [17–19] and confirms their observation that certain normal human sera may contain antibodies directed against antigenic determinants present in the cytoplasm of keratinocytes in the upper layers of the epidermis (U-Cyt antigens). This U-Cyt reactivity was not detected when NHS was replaced by PBS, or when the incubation with NHS was followed by antisera to human IgM, IgA or fibrinogen.

Detection of C3-binding Sites in Normal Human Skin

The incubation of cryostat sections of normal human skin with all normal human sera tested followed by FITC-R/Hu-C3 revealed bright cytoplasmic staining of different cell types within the epidermis and dermis. Virtually no fluorescent staining was seen when skin sections exposed to NHS were first incubated with unlabeled R/Hu-C3 before being reacted with FITC-R/Hu-C3. Keratinocytes of the upper epidermal layers displayed an homogenous, occasionally granular, strong cytoplasmic fluorescence, whereas nuclei, intercellular spaces and basal cell layers were consistently devoid of staining (Fig 1*a* and *b*). In addition, there was a very distinct cytoplasmic staining of cells displaying a polygonal or dendritic shape within the dermis. These cells were particularly abundant in the papillary dermis (Fig 1*b*), whereas in the deeper layers they were more sparse and scattered between bundles of connective tissue (Fig 1*c*). On the basis of distribution and shape, these cells were considered to represent fibroblasts. A third population of cells within normal human skin, the smooth muscle cells of musculi arrectores pilorum, also displayed bright cytoplasmic fluorescence (Fig 2*a*). Preliminary immunoelectron microscopic studies confirmed the intracytoplasmic binding of C3 in the three described cell populations and suggested that intermediate-sized filaments are the C-binding cytoplasmic structures (Schuler et al, manuscript in preparation).

Whereas the reactivity of the cytoplasm of these 3 cell types was detected consistently after incubation with each of the 20 normal test sera, positive staining of certain other cell types was observed only in a few instances. With 2 of the sera tested, a group of dendritic cells with bright cytoplasmic fluorescence was seen along the dermal-epidermal junction (Fig 3*a*). Counterstaining of tissue sections with ethidium bromide revealed that these dendritic cells were located within the basal layer of the epidermis; they were therefore considered to represent melanocytes (Fig 3*b*) and, indeed, the melanocyte nature of these cells has been confirmed by preliminary immunoelectron microscopic investigations (Schuler et al, manuscript in preparation). In addition, a cytoplasmic staining of cells along vascular structures was seen with eight of the test sera. Due to the limitations of immunohistologic techniques we were not able to determine whether positively stained cells in the vicinity of or along vascular structures represented endothelial cells, smooth muscle cells within the vessel wall or pericytes (Fig 1*d*).

These results suggested, but did not prove, that C3 deposition on cytoplasmic structures of different cell types can occur in the absence of circulating antibodies. When sera from patients with hereditary C4-deficiency were used instead of normal human sera, FITC-R/Hu-C3 failed to stain the cytoplasmic structures in question (Fig 4). This finding suggested that C3 deposition on the respective structures is a consequence of classical C pathway activation. The validity of this assumption was further substantiated by our finding that in the presence of EDTA or EGTA in the test sera at concentrations (10 mM) which allow blockade of the classical C pathway no staining reaction with FITC-R/Hu-C3 was disclosed. In control sections, when test sera were replaced by PBS, no specific reactivity was detected.

Detection of Clq-binding Sites in Normal Human Skin

The incubation of tissue sections with either NHS or purified Clq followed by FITC-R/Hu-Clq led in both instances to identical results: Strong cytoplasmic staining within keratinocytes of the upper epidermal layers (Fig 5*a*) and within smooth muscle cells of musculi arrectores pilorum (Fig 2*b*), indistinguishable from that seen with FITC-R/Hu-C3 was observed. However, within the dermis, a staining pattern was revealed which slightly differed from that seen after incubation with FITC-R/Hu-C3. Although cells, displaying a polygonal and frequently dendritic shape were clearly visualized, they were diffusely scattered within the dermis (Fig 5*b*) and were not

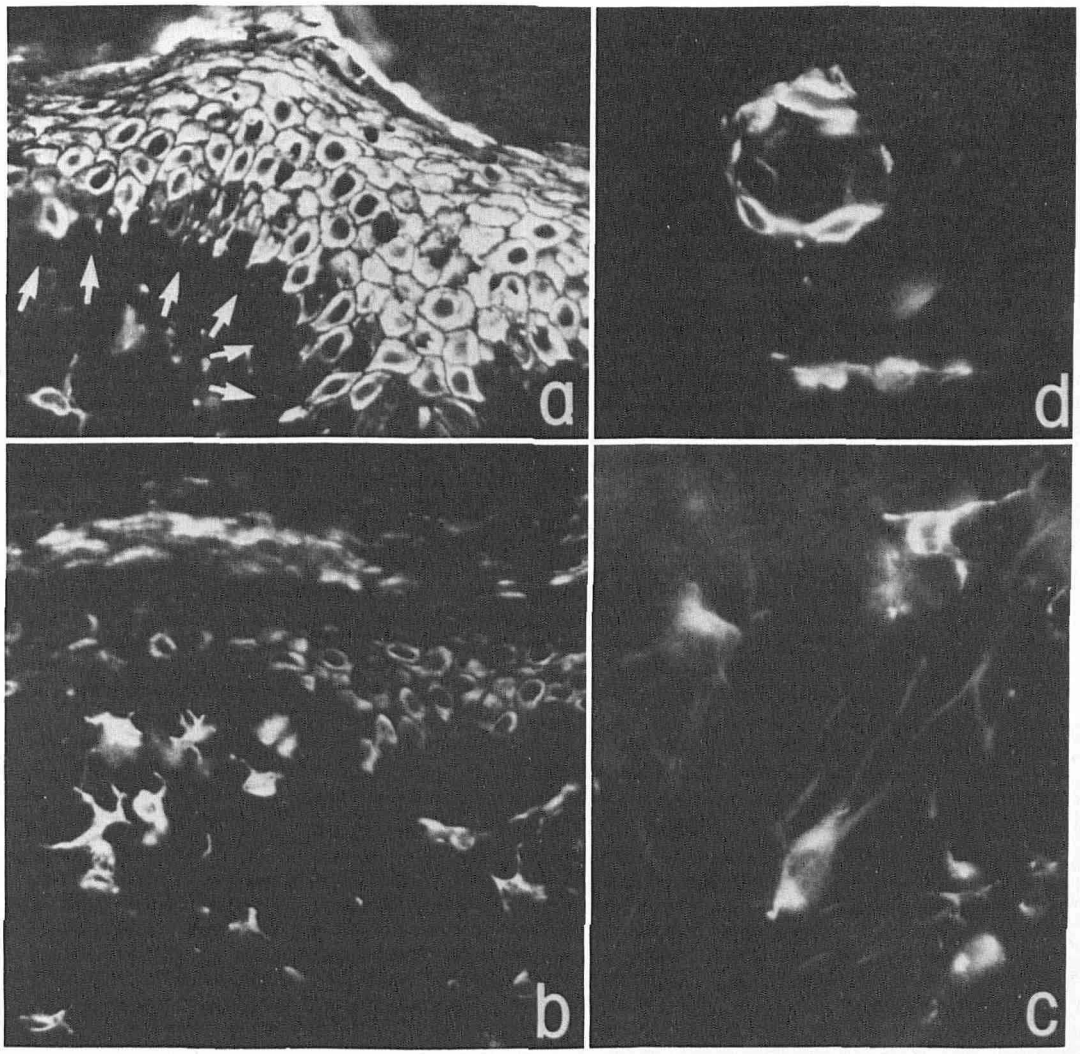

FIG 1. Cryostat sections of normal human skin were incubated with NHS and in a second incubation period exposed to FITC-R/Hu-C3: (*a*) Bright cytoplasmic staining of keratinocytes of the upper epidermal layers is seen. Nuclei and intercellular spaces as well as basal epidermal cell layers are devoid of staining. The dermal-epidermal junction is marked by *white arrows*. Note (lower left corner) one cell in the papillary dermis with bright cytoplasmic staining including dendritic processes (× 220). (*b*) In addition to the staining of upper epidermal keratinocytes highly dendritic cells clustered in the papillary dermis (fibroblasts) exhibit a strong cytoplasmic staining (× 220). (*c*) High-power magnification of brightly stained dendritic dermal cells scattered between bundles of connective tissue (× 440). (*d*) Cytoplasmic staining of cells outlining a medium-sized dermal vessel (× 440).

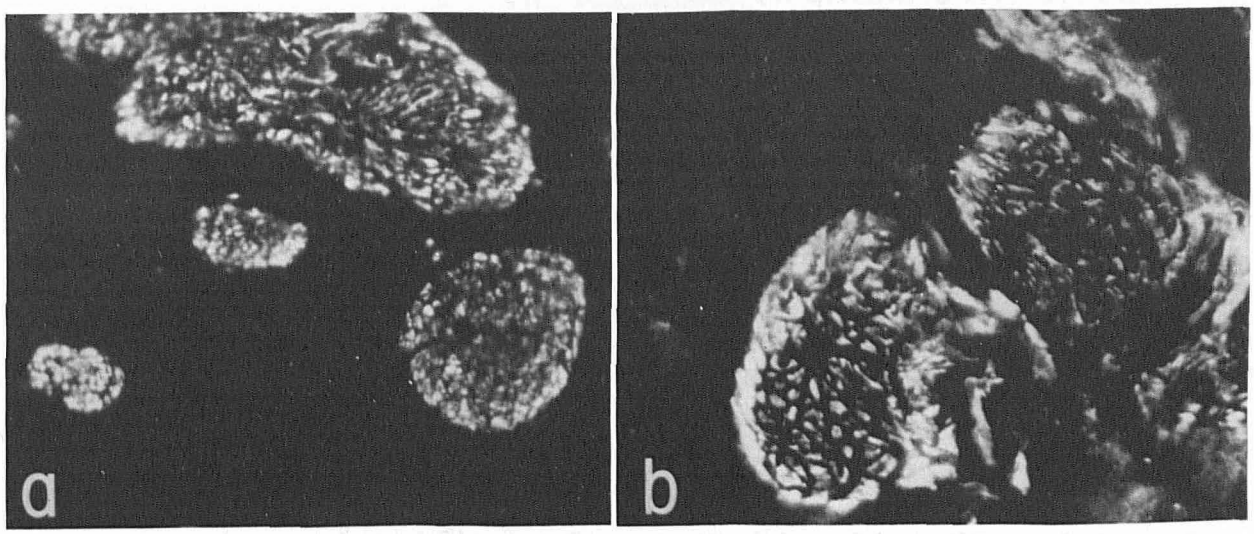

FIG 2. *a*, Cryostat skin sections were exposed to NHS and then to FITC-R/Hu-C3: bright staining of cross or oblique cut musculi arrectores pilorum is seen (× 220). *b*, Tissue specimens were first incubated with purified Clq and then with FITC-R/Hu-Clq: an identical staining pattern as that seen in Fig 2*a* is disclosed (× 220).

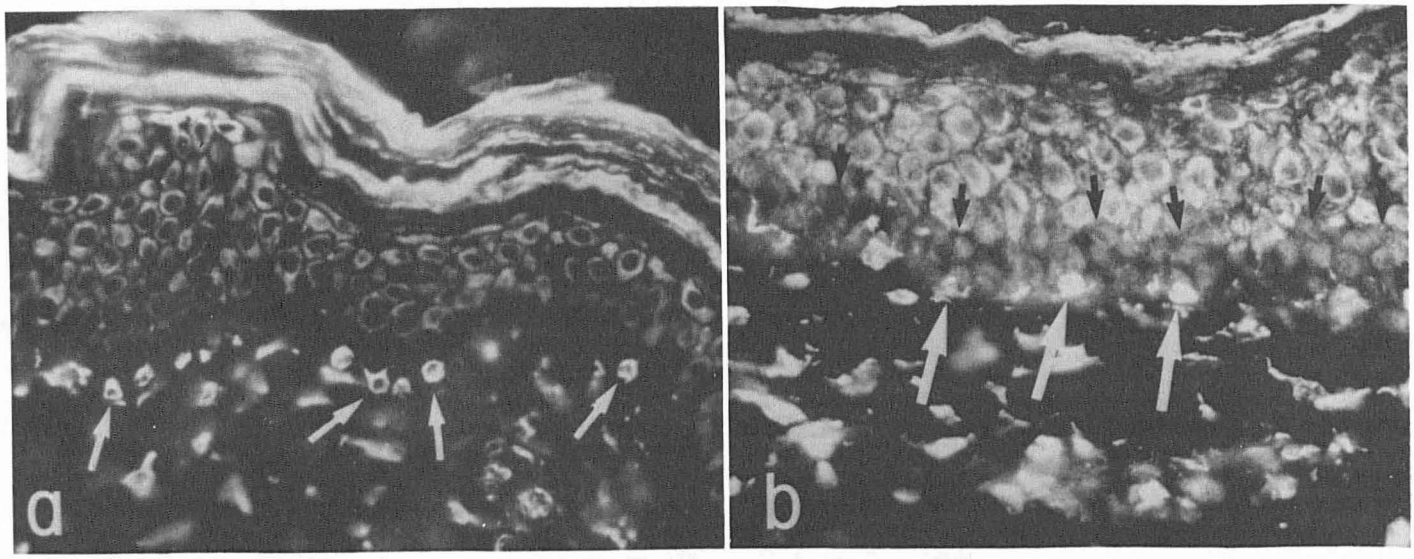

FIG 3. *a*, Cryostat sections of normal human skin were incubated with NHS followed by FITC-R/Hu-C3: Cells with bright cytoplasmic fluorescence (marked by white arrows) are detected along the dermal-epidermal junction (× 220). *b*, Counterstaining of cell nuclei with ethidium bromide reveals that cells described in Fig 3*a* are located within the basal cell layer of the epidermis (*white arrows*). *Black arrows* mark the border between keratinocytes of the upper layers exhibiting cytoplasmic staining and unstained basal keratinocytes (× 220).

FIG 4. A normal human skin cryostat specimen was incubated with the serum of a patient with hereditary C4 deficiency and then with FITC-R/Hu-C3: no specific staining within the epidermis or dermis is seen (× 90).

clustered along the dermal-epidermal junction as were the cells identified by FITC-R/Hu-C3. In addition, incubation with FITC-R/Hu-Clq disclosed a heavily stained cell population outlining vessel lumina which, most likely, represent endothelial cells (Fig 5*b*). Dendritic cells within the basal layers (melanocytes) escaped detection with any of the test sera or tissue sections employed.

It must be mentioned at this point that both endothelial cells and a few polygonal cells randomly distributed within the dermis, but not epidermal cells or smooth muscle cells were also stained, when control tissue sections were initially exposed to PBS instead of NHS or purified Clq and then reacted with FITC-R/Hu-Clq.

DISCUSSION

Using indirect IF techniques, Linder, Lehto, and Stenman have recently been able to show that cytoplasmic structures, most likely intermediate-sized filaments, present in human embryonal fibroblasts [8], in the endothelia of vessel walls [9,10] as well as in various muscle cells [9,10], have the capacity to bind complement components Clq, C4 and C3 when exposed to NHS. Furthermore, these authors presented suggestive evidence that binding of Clq to the respective cytoplasmic structures is the initiating event for further activation of the classical C pathway. In this report, we have searched for the presence of C-binding cytoplasmic structures in normal human skin and have made the following observations:

1. Exposure of cryostat sections of normal human skin to fresh NHS leads to the binding of C3 to cytoplasmic structures in suprabasal keratinocytes, fibroblasts and smooth muscle cells, frequently in cells associated with vascular structures and occasionally in melanocytes.

2. This C3-binding appears to be due to the activation of the classical C cascade in that it cannot be detected when either C4 deficient serum is used instead of NHS or when EGTA or EDTA at concentrations which inhibit complement activation by chelating Ca^{++} and Mg^{++} are added to the test serum during incubation.

3. Exposure of cryostat skin sections of normal skin either to fresh NHS or purified Clq followed by FITC-R/Hu-Clq also reveals cytoplasmic staining of upper keratinocytes, dermal cells, smooth muscle cells of musculi arrectores pilorum and endothelial cells, whereas incubation with FITC-R/Hu-Clq alone stains only endothelial cells and some dermal cells.

Among the cell types having cytoplasmic C-binding structures, 2 virtually identical populations were identified either by the use of FITC-R/Hu-C3 or FITC-R/Hu-Clq, i.e., keratinocytes of the upper epidermal layers and smooth muscle cells within musculi arrectores pilorum. Our data strongly suggest that these cell populations possess cytoplasmic structures capable of antibody-independent binding of Clq and that Clq binding initiates activation of the classical C cascade. This conclusion is based on the following observations: (a) incubation of tissue sections with purified Clq followed by FITC-R/Hu-Clq reveals staining which is not seen after incubation with FITC-R/Hu-Clq alone (Fig 2*b*, 5*a*); (b) incubation of tissue sections with NHS or C4-deficient serum followed by FITC-R/Hu-Clq gives an identical staining pattern; (c) incubation of tissue sections with NHS followed by FITC-R/Hu-C3 yields identical results whereas replacement of NHS by C4-deficient serum or performance of the IF assay in the presence of EGTA or EDTA yields negative results.

The staining pattern observed in suprabasal keratinocytes remarkably resembled that seen when cryostat sections of

normal human skin are reacted in an indirect IF procedure with normal human sera containing IgG antibodies against upper epidermal cytoplasmic antigens as described by other authors [17–19]. One may therefore argue that the C3- and Clq-binding observed in the cytoplasm of these cells may be due to antibodies directed against U-Cyt antigens which, because of a possible lack of sensitivity of the IF procedure, escape detection by incubation with FITC-anti-human IgG, but can be readily visualized by their C-binding capacity. Although we cannot absolutely exclude this possibility, we have shown that purified Clq in the absence of serum binds to cytoplasmic structures within keratinocytes of the upper layers and within smooth muscle cells which demonstrates that the binding of the molecule which initiates classical complement activation can occur independently of the presence of antibodies.

On the other hand, the consistent detection of C3 binding structures within the cytoplasm of dendritic fibroblasts of the papillary dermis (Fig 1*b*) and the occasional visualization of C3 within melanocytes and endothelial cells of small and medium-sized vessels may be due to a different mechanism. With all test sera and tissues used, large numbers of dendritic fibroblasts located in the upper dermis and, thus, in close proximity to the dermal-epidermal junction were seen after incubation of tissue sections with NHS followed by FITC-R/Hu-C3 (Fig 1*b*). These cells were not seen under conditions which do not allow activation of the classical complement cascade which excludes the possibility that the C3 binding may have occurred directly or due to alternative pathway activation. Surprisingly, however, these fibroblasts in the papillary dermis partly escaped detection when tissue sections were exposed to either purified Clq or NHS followed by FITC-R/Hu-Clq, although these reagents did reveal dendritic cells in the lower dermis (Fig 5*b*). Similarly, the melanocyte fluorescence seen after incubation of skin sections with certain NHS and FITC-R/Hu-C3 was not observed when FITC-R/Hu-C3 was replaced by FITC-R/Hu-Clq. We offer 2 possible explanations for this phenomenon. On one hand, both fibroblasts in the papillary dermis and melanocytes may have cytoplasmic Clq receptors in such small quantities that they escape detection by conventional IF but yet suffice to initiate C-activation and thus result in large numbers of bound C3 molecules, which can, at least in some instances, then be visualized by IF. Alternatively, it is conceivable that virtually all normal individuals possess circulating antibodies directed against cytoplasmic structures within fibroblasts which again—due to the limitations of IF procedures—can only be detected by their C-binding properties. In the case of melanocytes, Hertz et al [20] demonstrated antibodies against cytoplasmic determinants within melanocytes, nevus and melanoma cells in sera from 2 patients with a variety of autoimmune disorders via their C-fixing ability. It may well be that a few of our test sera also contained anti-melanocyte autoantibodies whose binding can only be visualized by their C-binding properties.

A third interesting observation was made when tissue sections were incubated with FITC-R/Hu-Clq alone. In tissue

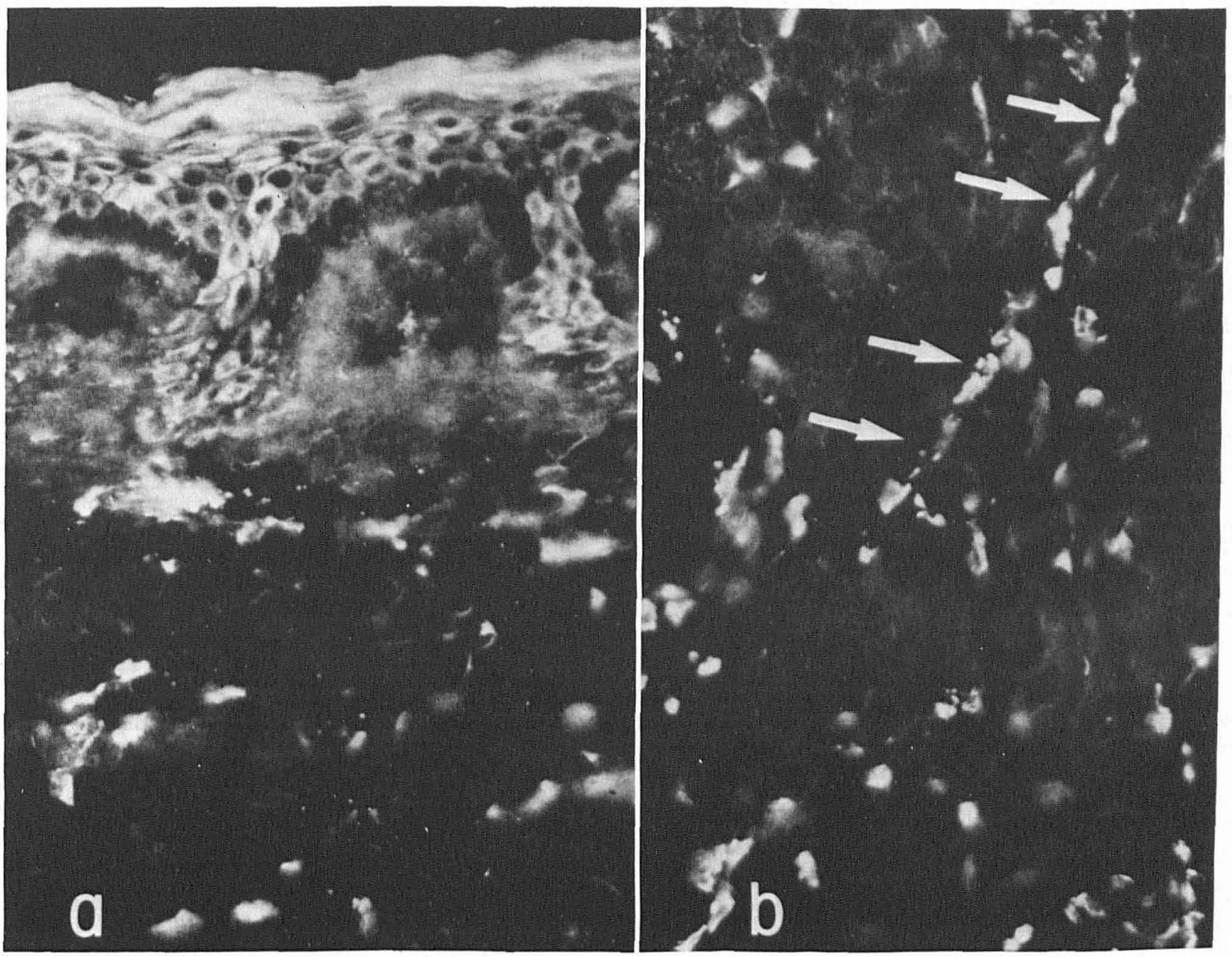

FIG 5. Cryostat skin sections are incubated first with purified Clq and then with FITC-R/Hu-Clq: *a*, The cytoplasm of epidermal cells within the upper Malpighian layers is stained; nuclei, intercellular spaces and basal epidermal layers do not show any specific staining (× 220). *b*, Cytoplasmic staining of cells, some possessing dendritic processes, within the reticular dermis. White arrows point to cells outlining vascular structures (× 220).

sections from all 10 different donors, a strong fluorescence of endothelial cells within small to medium-sized vessels and certain cells scattered between collagen bundles of the dermis was seen. The entire epidermis and smooth muscle cells were completely negative. The most likely interpretation of this finding is that the respective cells represent sites of Clq synthesis, or that complexed Clq has been taken up by those cells in vivo. This finding does not exclude the possibility that endothelial cells also possess cytoplasmic C-binding structures, although deposition of C3 within the cytoplasm of endothelial cells was not a regular finding and on a given substrate was only seen with 8 of 20 sera tested. Two possibilities may account for this finding. First, one might argue that Clq synthesized by endothelial cells does not initiate classical complement activation by itself but masks Clq binding structures to a varying extent thus allowing serum-derived Clq to bind and activate the classical C-cascade only in certain instances. This possibility is rather unlikely as the detection of cytoplasmic C3 binding was not substrate dependent but rather dependent upon the serum source used. Due to the careful handling of experimental serum samples, it appears unlikely that, as a consequence of degradation processes, the amount of single complement components available became the limiting factor. Alternatively, the role of C-fixing autoantibodies for the generation of cytoplasmic C3 binding within endothelial cells cannot be ruled out. Only recently it was reported that sera of patients with various inflammatory conditions contain antibodies which react with cytoskeletal intermediate-sized filaments of vascular endothelial cells [21]. Although our sera were derived from healthy individuals, the presence of only minute amounts of such antibodies in certain sera might suffice to allow their detection via their C-binding properties.

Another rather surprising finding deserves to be discussed in greater detail. Although Clq is reported to bind to DNA [7], we did not observe nuclear fluorescence when skin sections were incubated with Clq followed by FITC-R/Hu-Clq. Using Crithidia luciliae—a hemoflagellate which is widely used as a substrate for determination of serum antibodies against double-stranded DNA [22]—as a substrate, Linder [23] observed strong binding of Clq to kinetoplasts, (which consist essentially only of circular, double-stranded DNA) but only very slight Clq binding to Crithidia luciliae nuclei. These authors suggested that the lack of significant Clq binding to nuclei was due to a blocking of the Clq receptor on nuclear DNA by histones or other nuclear constituents. Although formal proof is lacking, this phenomenon may account for our inability to detect nuclear Clq binding.

Certainly, the most important observation of this study is that cytoplasmic structures exist in certain skin cells (keratinocytes, smooth muscle cells) which are capable of antibody-independent binding of Clq. Although formal proof is lacking our data strongly indicate that the binding of Clq to these respective cytoplasmic structures results in the activation of the classical complement pathway. Cell injury, as a consequence of either physical trauma, limited vascular supply, infectious and toxic agents or metabolic and immunological disturbances, would expose these cytoplasmic structures to Clq present within the interstitial fluid. With the binding of Clq the classical C pathway would be activated leading to the generation of biologically active split products of the C cascade. Since these peptides are mediators in a series of important biological mechanisms including immune adherence, acute inflammatory responses and can interact with other protein systems [24], the presented phenomena of C-binding and activation on cytoplasmic structures of normal human skin cells may be a critical event in the initiation of reparative processes which utilize the C-cascade and are operative in a variety of physiological and pathological processes affecting the skin.

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